REMARKS

1. Section 1 of the Office Action: Rejection of Claims 1-5, 7-18, 20-31,33-44, 46-52 and 67-76 under 35 USC §112(1)

As understood, the recited "monolayer" is regarded as new matter since a word search of the specification's text has failed to locate the terms "monolayer" or "layer". Kindly reconsider these rejections. The Court of Appeals for the Federal Circuit has long made it clear that there is no requirement that a claim recitation have *ipsis verbis* support in the specification to comply with §112(1) – in other words, the same language need not be used. As noted in *Kennecott Corp. v. Kyocera International Inc.*, 5 USPQ2d 1194, 1197 (Fed. Cir. 1987), both the CAFC (and its predecessor court, the Court of Customs and Patent Appeals) "has long recognized that an invention may be described in different ways and still be the same invention." The holding of *Kennecott* is then summarized in MPEP 2163.07(a):

> By disclosing in a patent application a device that inherently performs a function or has a property, operates according to a theory or has an advantage, a patent application necessarily discloses that function, theory or advantage, even though it says nothing explicit concerning it. The application may later be amended to recite the function, theory or advantage without introducing prohibited new matter. *In re Reynolds*, 443 F.2d 384, 170 USPQ 94 (CCPA 1971); *In re Smythe*, 480 F. 2d 1376, 178 USPQ 279 (CCPA 1973).[1]

In accordance with the foregoing cases, the teaching that DNA molecules bound to the surface of a solid support form a "monolayer" is plainly inherent in the application's specification, and thus does not constitute new matter. The application is directed to a hybridization system in which target DNA molecules are bound to a 2-D surface (as opposed to the 3-D matrix of the previously-

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[1] See also *Schering Corp. v. Amgen Inc.*, 222 F.3d 1347, 1352, 55 USPQ2d 1650, 1653 (Fed. Cir. 2000) ("The fundamental inquiry is whether the material added by amendment was inherently contained in the original application."); *TurboCare Div. of Demag Delaval Turbomachinery Corp. v. Gen. Elec. Co.*, 60 USPQ2d 1017, 1023 (Fed. Cir. 2001) ("In order for a disclosure to be inherent, 'the missing descriptive matter must necessarily be present in the application's specification such that one skilled in the art would recognize such a disclosure.') (quoting *Tronzo v. Biomet*, Inc., 156 F.3d 1154, 1159, 47 USPQ2d 1829, 1834 (Fed. Cir. 1998)); *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991) ("[T]he test for sufficiency of support... is whether the disclosure of the application relied upon 'reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter.'") (quoting *Ralston Purina Co. v. Far-Mar-Co, Inc.*, 772 F.2d 1570, 1575, 227 USPQ 177, 179 (Fed. Cir. 1985)).

cited *Drobyshev* reference), and the specification describes, for example, the use of a streptavidin/biotin interaction to attach the DNA to the surface (see, e.g., page 7 lines 15-26; page 11 lines 8-19; page 13 lines 18-19; etc.). These passages would be clearly understood by a skilled person to describe the recited "monolayer," since DNA molecules bound to a solid surface in this way *necessarily form a monolayer.* This is not a matter of probability or possibility; this is an inherent property of the system described in the application, and would be immediately recognized by persons of ordinary skill. To demonstrate, see for example *P. Peluso et al.*, Analytical Biochemistry 312 (2003) 113-124 (a copy of which accompanies this Response), which notes that matter bound to a surface in the manner described in the specification rests in a monolayer; see, e.g., the Abstract starting at the fifth sentence:

> We compare randomly versus specifically oriented capture agents based on both full-sized antibodies and Fab' fragments. Each comparison was performed using three different antibodies and two types of *streptavidin-coated monolayer surfaces.*

(Emphasis added.) To further illustrate, this Response is accompanied by a Declaration of Dr. Pavel Strohner, an artisan skilled at streptavidin surface coatings and inventor/owner of U.S. Patent 6,270,983 ("Surfaces Coated With Streptavidin/Avidin"). Dr. Strohner's Declaration states (see particularly Paragraph 5 of the Declaration):

> DNA bound to a solid surface by a streptavidin/biotin link would be considered in the field of DNA hybridization to be a monolayer of DNA molecules.

It is therefore seen that the feature of a "monolayer" of single DNA strands is necessarily present in the hybridization system described in the specification, and would be so recognized by persons of ordinary skill. Therefore, recitation of this feature does not introduce new matter. Kindly withdraw the rejections.

2. Section 2 of the Office Action: Priority

The parent/priority applications GB9821989.2 and PCT/GB99/03329 describe the same system as disclosed in the present application. Thus, for the reasons noted in the foregoing Section 2 of this Response, both of these priority documents provide §112(1) support for the term "monolayer," which is sufficient to accord priority in accordance with §119/§365 (see MPEP

201.11). Please reacknowledge that the current claims receive the priority from both parent applications.

3. Sections 3-4 of the Office Action: Rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52 and 67-71, 73, 74 and 76 under 35 USC §103(a) in view of *Stimpson et al.* (1995) PNAS 92:6379-6383 and U.S. Patent 6,174,670 to *Wittwer*

Kindly reconsider these rejections as well. *Stimpson et al.* describes a rapid detection method using a two-dimensional optical waveguide that allows measurement of real time-binding of a light-scattering label on a DNA array. A selenium antibody conjugate is used to label a biotinylated probe. Hybridization of the probe on the surface concentrates the selenium in close proximity to the waveguide, allowing light scattering to occur when light is injected into the waveguide using a slit. Light scattering is observed visually or with a CCD camera.

The Examiner then asserts that it would be obvious to one of ordinary skill to employ the SYBR Green markers of *Wittwer* in *Stimpson*'s mutation method to attain the claimed method "since *Wittwer* teaches that this intercalator [SYBR Green] is superior in sensitivity, is useful in the particular assay employed by Stimpson since the waveguides would detect the fluorescent label and is inexpensive" (Page 7, Office Action). However, closer review of *Wittwer* and *Stimpson* show that for the following reasons, an ordinary artisan would not in fact receive any objectively ascertainable motivation from either document to employ the markers of *Wittwer* in the mutation detection method of *Stimpson*.

4.a. The Markers of *Wittwer* Could Not Be Successfully Utilized In The Mutation Detection Method of *Stimpson et al.*

Stimpson et al. describes an original approach to hybridization detection that does not employ fluorescence, and is explicitly stated to be an advantageous alternative to fluorescence-based systems. To illustrate, *Stimpson* notes:

> The use of the optical wave guide readout has significant advantages over fluorescence readout with confocal microscopy (12), which is regarded as the current "gold standard".

(P. 6383, second paragraph of "Discussion.") Further, *Stimpson* emphasizes that a significant advantage of the optical waveguide approach over fluorescence-based systems is the ability to follow hybridization events in real time; for example:

> The signal intensity is sufficient to allow measurement of the surface binding and desorption of the light scattering label can be studied in real-time; i.e., detection is not rate limiting.

(P. 6379, near end of second full paragraph of second full column.) An artisan would thus understand from *Stimpson* that the optical waveguide system has significant advantages over fluorescent systems, and would not sacrifice these advantages by replacing the optical waveguide detection system with a fluorescence detection system employing SYBR Green 1: since there is no benefit to the asserted modification, there is no motivation to make it. *Stimpson* in fact states that there are significant disadvantages with fluorescence-based detection systems:

> Because the amount of fluorescent label on the surface is quite low, the time required to scan the array is on [sic] the order of 1 min. Such integration times are also typical for cooled CCD camera systems. Much higher DNA densities can be achieved by using a gel matrix and in this case the fluorescent signal can be read at a standard speed with a low sensitivity CCD camera. However, the gel system affects the kinetics of hybridization /melting through multiple binding events in the three dimensional matrix of immobilized DNA and requires a washing step.

In other words, detection of fluorescence on a solid surface is slow and cannot be performed in real-time, unlike *Stimpson*'s optical waveguide system. *Stimpson* further emphasizes the problems of producing melting curves using fluorescence-based methods:

> However, if 1 min is required to read/wash a DNA chip, then a high resolution melting curve from 30 to 70°C would require 40 min; i.e. measurement is rate limiting. Removal of background would require some sort of wash system to eliminate the label as it dissociates from the capture site.

In other words, *Stimpson specifically teaches that melting curves cannot be produced using DNA on a solid surface using fluorescence detection in real-time*. If an artisan were to replace the *Stimpson* optical waveguide scattering detection system with a fluorescence-detecting system, then the modified system would be unable to produce melting curves in real-time – and the objectives of *Stimpson* would thereby be obviated. Such a modification cannot be obvious since there is plainly no true motivation for an ordinary artisan to pursue it; see MPEP 2143.01, subsection

entitled "The Proposed Modification Cannot Render The Prior Art Unsatisfactory For Its Intended Purpose". Such a modification is also unobvious because it would completely change the principle of operation of the *Stimpson* system (from optical waveguides to fluorescence detection), and would incur all the disadvantages of fluorescence based detection noted by *Stimpson*. See MPEP 2143.01, subsection entitled "The Proposed Modification Cannot Change The Principle of Operation Of A Reference".

Further, to support the rejections, the Examiner asserts that "the waveguides would detect the fluorescent label". With respect, this is simply incorrect. *Stimpson et al.* employs selenium colloids of about 0.2 micrometers as light scattering particles (see P. 6380 at "Selenium Colloid and Conjugate"). A small organic molecule would not reasonably be expected to scatter light in the same way as a colloidal particle specially selected for its reflectivity. Neither could the "waveguides detect the fluorescent label" by more conventional fluorescence analysis, because *Stimpson et al.* explicitly states that this does not work. There is no reasonable expectation that the substitution asserted by the Examiner would have any reasonable expectation of success, as required by MPEP 2143.02.

To further illustrate the unobviousness of the claimed system, this Response is accompanied by a Declaration of Professor John D. Baldeschwieler; note that he was the senior investigator on the *Stimpson et al.* paper. Prof. Baldeschwieler indicates that no artisan would in fact find any reason to make the modification asserted by the Examiner; for example (at Paragraph 4):

> Given the well-known problems with fluorescence based assays on a solid surface, a skilled person in the field would not have been motivated to replace the signal generation mechanism described in Stimpson et al with any kind of fluorescence-based system.

In summary, since *Stimpson et al.* cannot be modified as asserted without negating its objectives and principles of operation; since there is no reasonable expectation that the asserted modification would lead to a successful result; and since no ordinary artisan would in fact be motivated to make the asserted modification (as indicated by the *Stimpson et al.* investigators themselves), kindly withdraw the rejections.

4.b. When Reviewed Objectively For All It Suggests, *Wittwer* Cannot Be Said to Fairly Suggest Use of SYBR Green I in The Mutation Detection Method of *Stimpson et al.*

In support of the rejections, the Examiner asserts that "Wittwer states that SYBR Green 1 is a preferred double strand specific dye for fluorescence monitoring of PCR" (Office Action, pages 6-7). *Wittwer* uses SYBR Green to monitor the production of dsDNA in PCR reactions. See column 40 at lines 48-53:

> Melting peaks can distinguish specific products from non-specific products (FIG. 40) and they can distinguish two purified PCR products mixed together (FIG. 41) so they should also be useful for distinguishing two specific products amplified together in a single reaction tube.

However, monitoring of PCR amplification is a very different process than the solid phase hybridization detection as described in *Stimpson* and the present application. Amplified DNA molecules in a PCR reaction are longer, free in solution and highly concentrated relative to the oligonucleotides immobilized on a solid surface. A skilled artisan is not taught by *Wittwer* that SYBR Green is useful in solid surface hybridization, which in no way suggests such use. Nor is SYBR Green used in *Wittwer* in any method of detecting single base sequence variations. Indeed, column 42 lines 53-59 state:

> When sequence specific detection and quantitation are desired, resonance energy transfer probes can be used instead of double strand specific DNA dyes. The Tm of hybridization probes shifts 4-8°C if a single base mismatch is present. If a hybridization probe is placed at a mutation site, single base mutations are detectable as a shift in the probe melting temperature.

In other words, if a skilled artisan wants to perform sequence specific detection and/or quantitation, *Wittwer* tells them to use labeled hybridization probes and FRET assays. *Wittwer* contains absolutely no mention or suggestion that relates to the use of SYBR Green for detection of DNA variation; if it is believed that a suggestion exists, kindly identify it for the record so that the Applicant may better respond.[2] Reading a teaching of the broader applicability of SYBR

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[2] "[W]hen the PTO asserts that there is an explicit or implicit teaching or suggestion in the prior art, it must indicate where such a teaching or suggestion appears in the reference," *In re Rijckaert*, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (citing to *In re Yates*, 211 USPQ 1149, 1151 (CCPA 1981)); "When relying on numerous references or a modification of prior art, it is incumbent upon the examiner to identify some suggestion to combine references or make the modification," *In re Mayne*, 41 USPQ2d 1451, 1454 (Fed.

Green I into *Wittwer* requires hindsight knowledge of the present invention, since there is nothing in *Wittwer* itself which would teach a skilled person to use SYBR Green for discriminating the melting curves of short immobilized oligonucleotides in the detection of DNA variation. If an artisan were to attempt this course, he/she would be ignoring the teaching of *Wittwer* as noted above, and as exemplified in Examples 18-23. Any suggestion that an artisan could or would simply plug the SYBR Green dye into the *Stimpson* method on the basis of *Wittwer* and expect to successfully achieve allelic discrimination is unreasonable, and based on hindsight reconstruction of the claimed invention.

4.c. In Summary

For the foregoing reasons (and based on the aforementioned Strohner and Baldeschwieler Declarations), and additionally in view of the Applicant's prior arguments (and the prior-filed Mirzabekov and Kwok Declarations), it is submitted that there is no case of *prima facie* obviousness. Kindly withdraw the objections.

4. Section 5 of the Office Action: Rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, and 67-76 under 35 USC §103(a) in view of *Stimpson et al.* (1995) PNAS 92:6379-6383, U.S. Patent 6,174,670 to *Wittwer*, and U.S. Patent 6,048,690 to *Heller et al*

These rejections rely on *Heller* as teaching that biotin-streptavidin binding to surfaces is known, and assert that the use of a biotin-streptavidin linkage in a hybridization method of *Stimpson* which employs the intercalating dye of *Wittwer* would be obvious. Kindly reconsider.

Cir. 1997) (citing to *In re Jones*, 21 USPQ2d 1941, 1943 (Fed. Cir. 1992) and *Ashland Oil, Inc. v. Delta Resins & Refractories, Inc.*, 227 USPQ 657, 664 (Fed. Cir. 1985)); see also *In re Rouffet*, 47 USPQ2d 1453 (Fed. Cir. 1998) (reversal of rejections for failure of Examiner and Board to "explain what specific understanding or technological principle within the knowledge of one of ordinary skill in the art would have suggested the combination", *Id.* at 1458; "even when the level of skill in the art is high, the Board must identify specifically the principle, known to one of ordinary skill, that suggests the claimed combination. . . . In other words, the Board must explain the reasons one of ordinary skill in the art would have been motivated to select the references and to combine them to render the claimed invention obvious", *Id.* at 1459). See also 37 CFR §1.104(c)(2).

Initially, *Heller et al.* describes the immobilization of biotinylated capture sequences in an avidin/agarose permeation layer, hybridization of these sequences with fluorescent labeled probes, and electronic denaturation of the hybridization complexes using a Pt electrode. *Heller* employs electronic perturbation for denaturation, and does not teach that a biotin/streptavidin linkage is sufficiently stable at high temperatures for producing a nucleic acid melting curve. There is no identification of any prior art which shows or fairly and objectively suggests use of a biotin/streptavidin linkage in a thermal denaturation method as presently claimed.

In addition, note that the distance between the selenium conjugate and the glass slide is important in the method of *Stimpson* in order for light scattering to occur, as the evanescent wave only extends 100-300 nm into the solution above the waveguide. *Stimpson et al.* fix the capture sequence to the slide via a chemical linkage. Replacement of this linkage with a biotin/streptavidin linkage, even if stable, would increase the distance between the selenium conjugate and the waveguide and would reasonably be expected to reduce or abrogate the light scattering signal produced by the selenium. Biotin/streptavidin is therefore not an "equivalent" of the chemical linkage which is employed in *Stimpson*, as asserted by the Examiner.

In addition, *Heller* fails to remedy the deficiencies of the teachings of *Wittwer* and *Stimpson* as described in the foregoing Section 4 of this Response, in particular since *Heller* is explicitly relied on solely to teach a biotin/streptavidin linkage.

For the foregoing reasons, a skilled artisan would not fairly find any motivation in *Heller* to employ a biotin/streptavidin linkage in the manner described by the Examiner. Kindly withdraw the rejections.

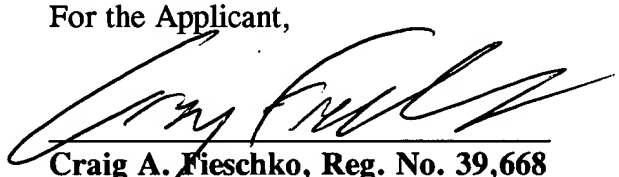
5. Section 6 of the Office Action: Rejection of Claims 1-6, 8-19, 21-32, 34-45, 47-52, 67-71, 73, 74 and 76 under 35 USC §103(a) in view of *Stimpson et al.* (1995) PNAS 92:6379-6383, U.S. Patent 6,174,670 to *Wittwer*, and U.S. Patent 5,789,167 to *Konrad et al.*

These rejections should also be withdrawn because *Konrad* fails to remedy the deficiencies of the teachings of *Wittwer* and *Stimpson* as described in the foregoing Section 4 of this Response.

6. In Cl sing

If any questions regarding the application arise, please contact the undersigned attorney. Telephone calls related to this application are welcomed and encouraged. The Commissioner is authorized to charge any fees or credit any overpayments relating to this application to deposit account number 18-2055.

For the Applicant,



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ATTACHMENTS:

- *P. Peluso et al.*, Analytical Biochemistry 312 (2003) 113-124
- Declaration of Dr. Pavel Strohner
- Declaration of Declaration of Professor John D. Baldeschwieler

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To whom it concern

1) I am the inventor and owner of the patent:

Strohner, P. Immer, U.: Surfaces coated with Streptavidin/Avidin, US-Patent 6,270,983 (7th August 2001), European patent pending. Positive evaluted by European Patent Office.

on means and methods for coating solid surfaces with streptavidin for various downstream applications, including DNA analysis. I am also the founder/owner of the company Biotez, which for many years has had its major business activity in coating different types of solid monolayer surfaces with streptavidin. In this capacity, I offer the following Expert Declaration on issues pertaining to the physical properties of streptavidin-coating of solid surface and molecules bound to such surfaces through biotin-streptavidin interaction.

2. Regardless of coating procedure details, immobilization of streptavidin onto solid-surfaces (such as plastic microtiter plates and membranes) will result in a reactive streptavidin monolayer. DNA molecules which are bound to this reactive streptavidin monolayer will inevitably form a superimposed DNA monolayer

3. The term 'monolayer' indicates a molecular arrangement wherein a number of DNA molecules are all similarly aligned, all at approximately the same distance from the basal binding surface, and all available for chemical reactions (e.g., hybridization) with other molecules that may approach from one and the same direction only.

4. DNA monolayer structures have distinct physicochemical properties and low binding capacities (whether created by streptavidin-biotin linkages or any of a wide range of chemical bond arrangements) that are fundamentally different to those observed for DNA in solution or immobilized in 3-D arrangements in a gel matrix. Unlike in solution and in gels, reacting species cannot approach a DNA monolayer immobilized to a solid surface from all-around in 3-D space in an unhindered manner. DNA monolayers may also have increased target-target interactions and reduced binding/reaction capacity relative to DNA in solution or in gels.

5. In conclusion, it is my understanding that DNA bound to a solid surface by a streptavidin-biotin link would be considered in the field of DNA hybridization to be a monolayer of DNA molecules, with all that entails.

6. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature

Dr. Pavel Strohner

CEO

Date: 27th Nov. 2003

Docket Number: 40225.000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Brookes, et al.
Serial No.	:	09/755,747
Examiner	:	Jeffrey Fredman
Art Unit	:	1637
Filed	:	5 January 2001
For	:	DETECTION OF NUCLEIC ACID POLYMORPHISM

DECLARATION OF JOHN D. BALDESCHWIELER

1. I am a Professor of Chemistry at Caltech, and for many years I have researched into areas such as spectroscopy, microscopy, and the application of these and other molecular techniques to the study of biological systems. In that capacity, I offer the following Expert Declaration on issues pertaining to a 1995 paper on which I was the senior investigator (Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides. Stimpson DI, Hoijer JV, Hsieh WT, Jou C, Gordon J, Theriault T, Gamble R, Baldeschwieler JD. Proc Natl Acad Sci U S A. 1995 Jul 3;92(14):6379-83: 'Stimpson et al').

2. Specifically, the purpose of the stated publication was to establish a means for real-time tracking of DNA melting that would work effectively on a solid surface. Both before and after the publication of Stimpson et al, , one skilled in the art would not expect the DNA binding capacity of any of the stable and common 2-D surfaces and chemistries to yield sufficiently strong fluorescent signals sufficiently 'instantly' (sub-second) in a fluorescence based assay method to allow for dynamic tracking of signal changes in real-time, when applying practically useful rates of heating. One skilled in the art would, therefore, most rationally turn to 3-D (gel-type) arrays to solve this widely recognized problem, since the considerable 3rd dimension provides far greater capacity and scope for DNA binding and manipulation.

3. The known limitations of solid surface fluorescence assays (i.e. 2D surfaces) compared to gel type alternatives (i.e. 3D matrices), are repeated emphasized in the Introduction, Results, and Discussion sections of Stimpson et al . Indeed, these factors were a large part of the motivating force that led us explore the alternative signal generation mechanism that we describe in Stimpson et al. Our solution was to channel an evanescent wave of intense light through a wave-guide in such a way that it became scattered (and thereby externally detectable) only from those surface regions to which suitably modified duplex DNA strands were bound.

4. Given the well-known problems with fluorescence-based assays on a solid surface, a skilled person in the field would not have been motivated to replace the signal generation mechanism described in Stimpson et al with any kind of fluorescence-based system.

5. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature... John D Baldeschwieler Date.... 11/11/03

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Optimizing antibody immobilization strategies for the construction of protein microarrays

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Abstract

Antibody microarrays have the potential to revolutionize protein expression profiling. The intensity of specific signal produced on a feature of such an array is related to the amount of analyte that is captured from the biological mixture by the immobilized antibody (the "capture agent"). This in turn is a function of the surface density and fractional activity of the capture agents. Here we investigate how these two factors are affected by the orientation of the capture agents on the surface. We compare randomly versus specifically oriented capture agents based on both full-sized antibodies and Fab′ fragments. Each comparison was performed using three different antibodies and two types of streptavidin-coated monolayer surfaces. The specific orientation of capture agents consistently increases the analyte-binding capacity of the surfaces, with up to 10-fold improvements over surfaces with randomly oriented capture agents. Surface plasmon resonance revealed a dense monolayer of Fab′ fragments that are on average 90% active when specifically oriented. Randomly attached Fab's could not be packed at such a high density and generally also had a lower specific activity. These results emphasize the importance of attaching proteins to surfaces such that their binding sites are oriented toward the solution phase.

Keywords: Antibody immobilization; Oriented binding; Fab; Protein array; Streptavidin

Proteomics consists of the highly parallel analysis of the expression, functions, modifications, and interactions of proteins within an organism. The most common method for the simultaneous analysis of the expression levels of numerous proteins from biological samples is two-dimensional (2-D)[2] PAGE, which allows hundreds to thousands of proteins to be measured in a single experiment [1,2]. This technique is, however, restricted in its sensitivity, throughput and ease of use. By contrast, multiwell enzyme-linked immunosorbent assays (ELISA) are highly sensitive, quantitative, and procedurally simple, but can measure only one analyte in each well. Microarrays of antibodies or other "capture agents" could, in theory, combine the high sensitivity and throughput of ELISA-type methods with the multiplexed nature of 2-D PAGE [3–12].

Immunoassays require one of the antibodies to be immobilized onto a surface. This is most simply accomplished by noncovalent adsorption of the capture antibodies onto plastic, poly-lysine-coated glass or other surfaces. Numerous studies over the past 40 years have shown, however, that these types of procedures generally denature the majority of adsorbed proteins (reviewed in [13,14]). Alternatively, proteins can be covalently coupled to chemically activated surfaces through the reaction of

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[1] These authors contributed equally.
[2] *Abbreviations used:* 2-D, two-dimensional; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MEA, 2-mercapto ethylamine; NEM, *N*-ethylmaleimide; FPLC, fast-performance liquid chromatography; HRP, horseradish peroxidase; SA, streptavidin; ARP, *N*-(aminooxyacetyl)-*N*′-(D-biotinoyl)hydrazine; SPR, surface plasmon resonance; b-SAM, biotinylated self-assembled monolayer; RU, response units; RIU, refractive index units; PLL, poly-L-lysine; PEG, polyethylene glycol; PE, R-phycoenythrin; IL-2, interleukin-2; IL-8, interleukin-8.

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lysine side chains. Proteins can be directly coupled to amine-reactive surfaces [15–22] or they can be biotinylated using NHS-activated biotin and then immobilized onto streptavidin (SA)-coated surfaces [23]. Such methods may be less denaturing than random noncovalent adsorption, but the random nature of the attachment can cause some of the immobilized protein to lose binding activity due to (i) direct chemical modification of the antigen-binding site, (ii) steric hindrance by the surface itself, (iii) steric hindrance by adjacently immobilized antibodies, and (iv) denaturation due to strain from multiple attachment sites. For these reason, antibodies are sometimes specifically immobilized onto surfaces such that the antigen-binding site is oriented away from the surface itself [24]. Specific orientation can be accomplished by using an intermediate protein, itself directly coupled to the surface, that binds to the Fc region of antibodies [13,14,20,21,25,26]. The intermediate protein can be protein A or protein G or an Fc-binding antibody. This type of immobilization strategy can result in a significantly higher fraction of active antibodies, but usually suffers from a lower surface density of immobilized antibody since two immobilization steps are required [20,21]. This method may also suffer from instability of the antibody–intermediate protein interaction.

Another method for specifically orienting antibodies on surfaces consists of chemically modifying the carbohydrate on the Fc region so that it can be attached to a surface [16,20,27–31]. This type of immobilization leaves the Fab fragments free to interact with antigens. Alternatively, the antibody (IgG) can be cleaved with pepsin to remove the Fc domain [32–34], and subsequently the disulfide bonds that link the two Fab domains together can be cleaved by a mild reducing agent [35]. The resulting cysteine thiols, which are on the opposite side of the Fab′ fragment relative to the antigen-binding site, can then be used as chemical handles to attach the Fab′ fragments to surfaces [19,21–23,36,37]. In this way, the antigen-binding site on the Fab′ is positioned to face away from the surface. Both of these methods can increase the fraction of active antibody fragments.

In this study, we compare four different antibody immobilization strategies (Fig. 1), all based on the binding of biotinylated antibodies (or fragments thereof) to streptavidin-coated surfaces. The four types are randomly biotinylated IgG, oriented IgG (biotinylated on

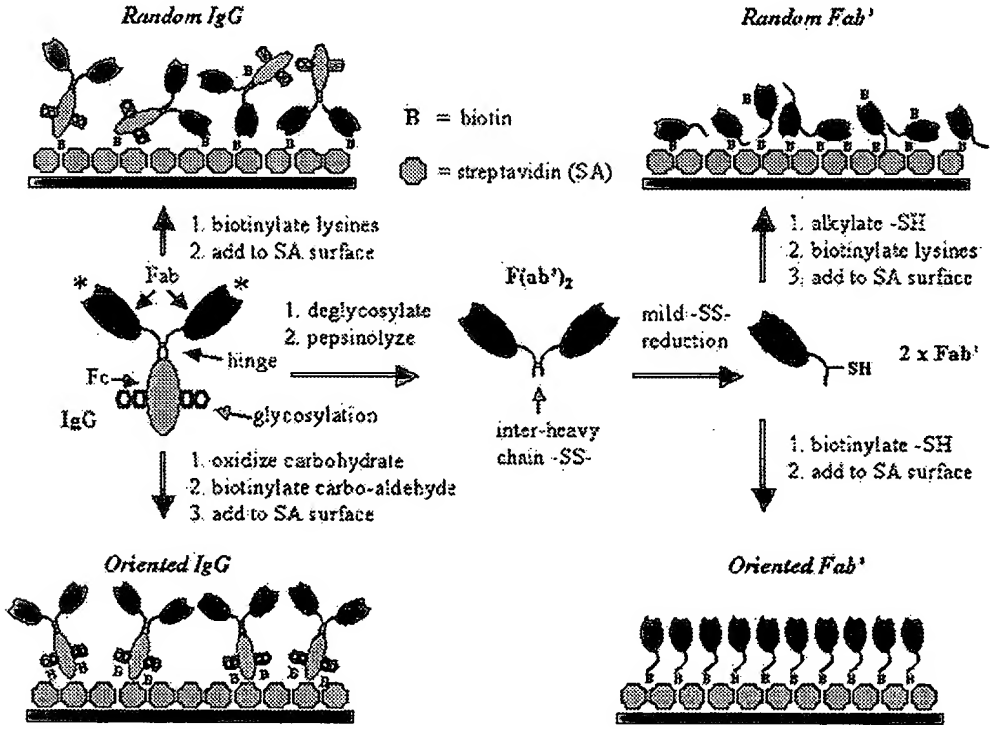

Fig. 1. Four immobilization strategies for the capture agents used in this study. The structure of the monoclonal IgG is shown in the left-middle portion of the diagram. It consists of two antigen-binding (Fab) fragments connected by a hinge region to the Fc portion, which is usually glycosylated. The antigen-binding face of the Fab's are marked by asterisks. The IgGs can be randomly biotinylated on lysine residues by NHS–biotin. To orient the IgGs, the conserved N-linked glycosylation site on the Fc portion can be oxidized with periodate and subsequently biotinylated using the biotin–aminooxy compound ARP. Alternatively, the Fc region can be removed by pepsinolysis and the resulting $F(ab')_2$ fragment is then reduced to monomeric Fab′ fragments. The reduced cysteine thiols on the hinge region can then be modified with biotin–maleimide, which allows for oriented attachment of the Fab′ fragments on the streptavidin (SA) surface. The Fab′ fragments were also alkylated and then randomly biotinylated, thus giving randomly oriented Fab′ fragments on the SA surface.

carbohydrate on Fc domain), oriented Fab′ fragments (biotinylated in hinge region), and randomly biotinylated Fab′ fragments. We compare these four preparations of three different antibodies against human cytokines with regard to surface density and binding activity. We also compare their antigen-binding performance in a protein microarray format.

Materials and methods

Materials

MAB9647 is a mouse IgG_1 that was raised against the human interleukin-8 (IL-8); it was produced by Covance (Princeton, NJ) from mouse ascites fluid using the hybrodoma cell line HB-9647 from ATCC (Manassas, VA) and is protein G purified. MAB208 is a mouse IgG_1 that was raised against human IL-8 by R&D Systems (Minneapolis, MN); it is protein G purified from mouse ascites fluid, Clone No. 6217.111; Cat. No. MAB208. MAB602 is a mouse IgG_{2A} that was raised against human interleukin-2 (IL-2) by R&D Systems; it is protein G purified from mouse ascites fluid; Clone No. 5355.111; Cat. No. MAB602. As a detection antibody for the IL-8 assays, an R-phycoerythrin (PE)-conjugated mouse anti-IL-8 antibody was used (BD Pharmingen, San Diego, CA; Cat. No. 20795A). For the IL-2 assays, two detection antibodies were used: a goat anti-human IL-2 antibody (R&D Systems; Cat. No. AF-202-NA) and an R-PE-conjugated donkey anti-goat IgG $F(ab')_2$ (Jackson Immuno Research, West Grove, PA; Cat. No. 705-116-147). Unless noted, chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Pepsin–agarose was purchased from Pierce (Rockford, IL; Product No. 20343). PNGase F was obtained from New England Biolabs (Beverly, MA; Product No. P0704). Aldehyde-reactive probe was from Molecular Probes (Eugene, OR). Human IL-2 was purchased from Leinco Technologies (St. Louis, MO; Product No. 011R455) and was reconstituted in phosphate-buffered saline (PBS; 11.9 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl; pH 7.4). A DNA sequence encoding the mature version of human IL-8 (AVLPRSAKELRCQCIKTYS KPFHPKFIKELRVIESGPHCANTEIIVKLSDGREL CLDPKENWVQRVVEKFLKRAENS) with a C-terminal Factor Xa cleavage site and Protein Kinase A site (GIEGRRRASV) was created by gene assembly of oligonucleotides. This construct was inserted into the *Nde*I and *Xho*I sites of pET24a (Novagen, Madison, WI), resulting in the further addition of a His(6) tag at the extreme C terminus (LEHHHHHH; where the LE codons comprise the *Xho*I site). This plasmid was then used for expression of IL-8 in BL21 (DE3) cells (Novagen) grown in EZMix modified Terrific Broth (Sigma; Cat. No. T-9179) by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside at an OD_{600} of 0.6 and growth for 4 h in a BioFlo3000 fermentor (New Brunswick Scientific, Edison, NJ) at 30 °C. The cells were collected by centrifugation, resuspended in 5 ml buffer/g with 300 mM NaCl, 50 mM sodium phosphate, 5 mM β-mercaptoethanol, 5 mM imidazole, pH 8.0, including one Complete Protease Inhibitor Cocktail tablet (Roche Applied Science; Cat. No. 1697498) per 50 ml, and lysed using a microfluidizer. The soluble fraction of IL-8 was purified by metal affinity chromatography using TALON Superflow beads (Clontech, Palo Alto, CA; Cat. No. 8908-2) followed by gel filtration in PBS using a Superdex 75 prep-grade column (Amersham Biosciences, Piscataway, NJ). The IL8-containing fractions were concentrated to 0.4–0.5 mg/ml using a Model 8400 stirred ultrafiltration cell (Millipore, Bedford, MA; Cat. No. 5124) with a 3K MWCO membrane and then dialyzed into PBS with 10% glycerol for storage. Identity and correct secondary structure were confirmed by mass spectrometry, ELISA, and circular dichroism (Spectrapolarimeter J-810; Jasco, Easton, MD).

Preparation of biotinylated Fab′ fragments

Because the conserved N-linked glycosylation of IgGs can inhibit cleavage by pepsin [34], the carbohydrate was removed from the antibodies under the following reaction conditions: 1–4 mg/ml antibody in 50 mM sodium phosphate, pH 7.5, 10–20 U/μl PNGase F (from New England Biolabs; using their unit definition), 24–48 h at 37 °C. After deglycosylation, antibodies were buffer-exchanged (using ultrafiltration or dialysis) into 20 mM sodium acetate (NaOAc), pH 4.5. Conditions for pepsinolysis were as follows: 30% (by volume) pepsin agarose (settled bed volume, beads washed in 20 mM NaOAc, pH 4.5), 0.5–2 mg/ml IgG, 20 mM NaOAc, 260 mM KCl, 0.1% Triton X-100, pH 4.5. Reactions were incubated at 37 °C with agitation for an amount of time that had previously been optimized (MAB9647, 12 h; MAB208, 4.5 h; MAB602, 3.5 h). After pepsin treatment, the fragments were recovered from the pepsin agarose by washing the resin with 0.1 M NaOAc, pH 4.5. The products of the pepsin cleavage were then concentrated and exchanged into 0.1 M sodium phosphate, 5 mM EDTA, pH 6.0, and then treated with 20 mM 2-mercaptoethylamine (MEA) in the same buffer for 90 min at 37 °C. The MEA was then removed by dialyzing for 6 h at 4 °C against 0.1 M sodium phosphate, 5 mM EDTA, using a 10-kDa cutoff membrane, and then residual MEA was removed by running the sample over a desalting column (PD-10; Amersham). Immediately after this step, the reduced Fab′ was treated with 20 mM *N*-ethylmaleimide (NEM) or maleimide-activated biotin (Pierce; Product No. 21901) for 2 h at room temperature, and the unincorporated NEM or

biotin–maleimide was then removed by dialysis. The samples were concentrated and the Fab′ fragments were purified from other fragments by FPLC using a Superdex-75 gel-filtration column (Amersham). In the case of the NEM-treated Fab′ fragments, random biotinylation was as described below.

Samples of the FPLC-purified Fab′ fragments were diluted 1:1 with nonreducing protein loading buffer (62.5 mM Tris–HCl, 25% glycerol, 2% SDS, 0.01% bromphenol blue) and loaded onto a 4–20% gradient SDS–polyacrylamide gel (Product No. 161-1123, Bio-Rad, Hercules, CA). SDS–PAGE was performed according to Laemmli [38]. In each case the ~50-kDa band corresponding to the Fab′ was observed, and the only observable contaminants correspond to the sizes of the free light and cleaved heavy chains. Because these contaminants comigrated with the Fab′ in a high-resolution gel filtration column, they presumably correspond to a noncovalent but otherwise structurally native complex between the free light and the cleaved heavy chains, in which the disulfide bond that normally links them has been reduced and alkylated. These fragments are likely to be functional since the disulfide bond between the heavy and the light chain is not in the antigen-binding site. They correspond to about 20% of the purified protein, and some of these Fab′ fragments are >80% active (see below).

The biotinylation of all capture agents used in this study was verified by Western blot analysis using an HRP-conjugated streptavidin probe (data not shown). In addition, the extent of biotinylation was estimated by using an SA resin pull-down assay (data not shown). Biotinylation was 60% or greater in each of these reactions. No attempt was made to remove nonbiotinylated protein prior to surface immobilization.

Carbobiotinylation of IgGs

IgGs can be site-specifically modified on the carbohydrate domain of the Fc region by oxidizing the vicinal diols to aldehydes then reacting them with hydrazide-derivatized surfaces [27–30] or with biotin–hydrazide, and subsequently attaching them to SA-coated surfaces [20]. We attempted the latter method, but found that the antibodies lost their biotin groups during storage at 4 °C. We therefore substituted biotin–hydrazide with *N*-(aminooxyacetyl)-*N*′-(D-biotinoyl)hydrazine [39] (ARP; Molecular Probes, Eugene, OR), which reacts with an aldehyde to form an *O*-alkyl oxime, which is more stable than the hydrazone linkage formed using biotin–hydrazide. This molecule was designed as a probe for abasic DNA sites. We find that glycoproteins biotinylated in this way remain biotinylated for >1 month at 4 °C (data not shown). Specifically, IgGs (3–5 mg/ml) were dialyzed into coupling buffer (0.1 M NaOAc, pH 5.5) and then incubated with 20 mM sodium metaperiodate in the dark for 1 h at 0 °C. The reaction was then quenched by the addition of 30 mM glycerol for 10 min, filtered to remove insoluble salts, and then dialyzed against coupling buffer for 6 h. ARP was then added at 1 mg/ml and incubated at room temperature for 2 h, after which the sample was extensively dialyzed against PBS.

Random biotinylation of IgG and Fab′ fragment molecules

IgG and NEM-treated Fab′ fragments were modified with the amine-reactive probe EZ-Link Sulfo–NHS–Biotin (Pierce). Reactions were performed with a 20-fold molar excess of biotinylation reagent over protein in PBS at room temperature for 2 h. The biotinylation reagent was then quenched by adding Tris–HCl, pH 7.4, to a final concentration of 10 mM. The samples were then dialyzed against a 1000-fold excess of PBS five times to remove free biotin probe. After dialysis, the biotinylated proteins were analyzed on a gel and tested for extent of biotinylation on UltraLink Plus Immobilized Streptavidin Gel (Pierce). Typically 60–100% of the protein was biotinylated (data not shown).

BIAcore studies to measure surface coverage and activity

All surface plasmon resonance (SPR) assays were performed on a BIAcore 3000 using a biotinylated self-assembled monolayer formed on a gold-coated glass surface by immersion in an ethanolic solution of an unsymmetrical alkanedisulfide. The omega-functionalities of the oligo(ethylene glycol)-containing alkane disulfides are *N*-hydroxysuccinimide and methoxy groups. This monolayer was then reacted with tri(ethylene glycol) amino biotin to give a biotinylated surface (unpublished). We refer to this surface as the biotinylated self-assembled monolayer (b-SAM). The biotin groups on the surface allow for the binding of SA. All assays were performed at 25 °C in PBS with 0.05% Tween 20. SA was loaded onto the surface at a flow rate of 20 μl/min at 0.1 mg/ml. Typically 320 μl was loaded to achieve a saturated surface of SA, whereby we typically obtained a surface coverage of 3.7–4.0 pmol/cm^2 (2.2–2.4 × 10^{12} molecules/cm^2), as calculated according to the equation [40]

$$\text{Surface Coverage} = [l_d/2][X/m(\eta_{\text{protein}} - \eta_{\text{solvent}})][\rho_{\text{protein}}] \times [1/\text{MW}],$$

where surface coverage is in moles/cm^2 and X is the Biacore signal shift as measured in Biacore response units (RU). For the calculations a value of 1.3 g/cm^3 was used for the typical density of a protein, ρ_{protein} [40]. The decay length of the evanescent wave, l_d, was assumed to be 3×10^{-5} cm as derived from Maxwell's equation [40]. The constant m represents the sensitivity of the BIAcore 3000, relating BIAcore response units to refractive index

units (RIU). A value of 9.7×10^5 RU/RIU was used according to BIAcore specifications and confirmed experimentally by calibration measurements. The typical refractive index of a protein, $\eta_{protein}$, was assumed to be 1.57 RIU [41]. The refractive index of solvent, $\eta_{solvent}$, was measured to be 1.335 RIU using an ABBE-3L Refractometer (Spectronic Instruments, Rochester, NY). For molar masses, values of 60,000, 150,000, 50,000, 11,200, and 15,400 g/mol were used for streptavidin, a full-length antibody, a Fab′ fragment, IL-8, and IL-2, respectively.

After SA deposition, the various capture agents were loaded at 20–100 nM at a flow rate of 20 μl/min until saturation was observed. For analyte (we use this term exclusively to refer to the cytokines) binding, flow rates of 80 μl/min were used unless otherwise noted. Various analyte concentrations over a broad range were assayed to determine the upper limit of analyte binding. For comparison, nonspecific binding of the analytes to SA was tested independently at all analyte concentrations studied.

Microarray assays

Microarray assays were performed on the biotinylated-poly-L-lysine (PLL)–polyethylene glycol (PEG) system as previously described [42,43] on a chip with six separate flow cells, each containing 250 addressable features (Fig. 2). The PLL has a molecular weight range of 15–30 kDa. Thirty percent of the PEG side chains (average length of 77 PEG units) are derivatized with biotin, whereas the remaining (average length of 44 PEG units) are methoxy-terminated. After SA deposition, the surfaces were washed and assembled into the flow cell chamber set up as described [42]. The surface-bound SA tetramers have from zero to three binding sites available to the biotinylated capture agents, depending on the number of bound PEG–biotins. The various capture agents tested were then loaded into different flow cells by applying typically 150 μl of a 100 nM stock of each capture agent over the appropriate flow cell at a flow rate of 0.05 ml/min. After extensively washing the flow cells with PBS, analyte was flowed over the cells at the indicated concentrations at a flow rate of approximately 20 μl/min and allowed to incubate for an additional 30 min.

For IL-8 assays, the analyte was diluted into Superblock/Tris-buffered saline (Pierce) with 0.05% Tween 20. Unbound analyte was rinsed out of the flow cell by applying 0.5 ml of PBS with 0.05% Tween 20 and 350 mM NaCl over the flow cells over a 5-min period. This was followed by addition of 200 μl of PE-conjugated anti-IL-8 secondary antibody (10 nM) over each flow cell in Superblock with 0.05% Tween 20 and 250 nM mouse IgG Fc as a blocking agent. The sec-

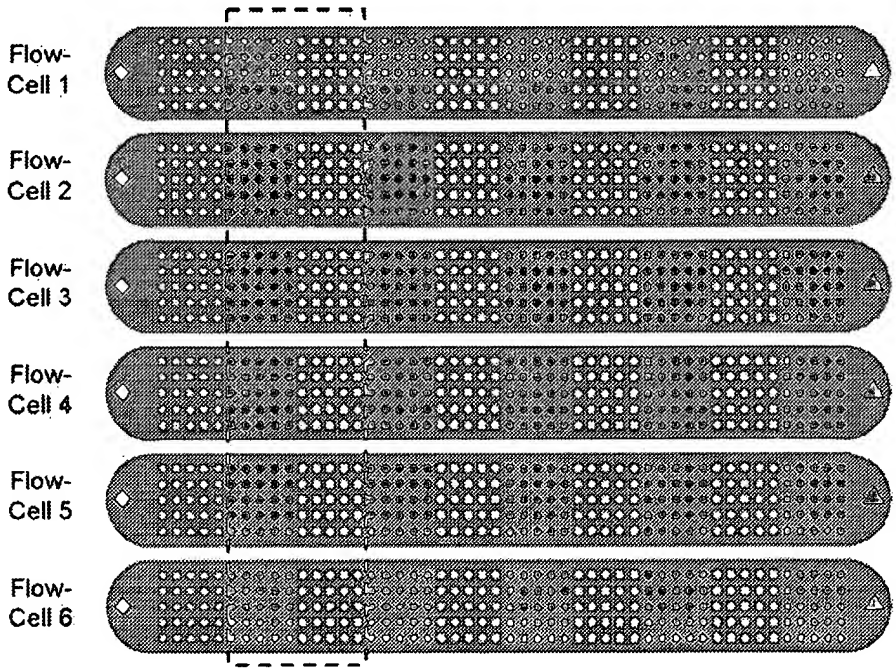

Fig. 2. Schematic diagram of the microarray device. The six individual flow cells are shown. Inlet (black diamonds) and outlet (black triangles) ports are at the left- and right-hand sides, respectively, of each flow cell. Within each flow cell are 250 features in a 5 × 50 array. Each flow cell has dimensions of 1.7 × 10 mm and holds about 8 μl of sample. The black and white circles represent features that are and are not derivatized with SA, respectively. For the experiments described here, each flow cell was filled with a solution of a single type of biotinylated capture agent, allowing for immobilization on the features of the 5 × 5 arrays that are SA-derivatized, but not on the adjacent 5 × 5 arrays. Different flow cells received different capture agent forms. After allowing immobilization to take place, the flow cells were washed. Next, analyte was added, followed by washing and detection of bound analyte as described in the text. The dashed line rectangle represents the portion of the chip that is shown in Figs. 5, 7, and 8.

ondary antibody was passed over the flow cell at a flow rate of 20 µl/min and allowed to incubate for an additional 30 min. The flow cells were rinsed with PBS with 0.05% Tween 20 and 350 mM NaCl over a 5-min period. The microarray chips were then removed from the flow cell apparatus and quickly rinsed with 25 ml of PBS followed by 10 ml of H_2O. The chips were then analyzed in a GSI Lumonics 5000XL microarray scanner and data were quantified using the QuantArray software (GSI Lumonics, Billerica, MA).

For IL-2 assays, the conditions were similar to those for IL-8, except that the analyte was in PBS with 3% nonfat milk and 0.05% Tween 20 during the incubation with the surfaces. In addition, the IL-2 assays were based on a tripartite antibody sandwich system which employed a goat-anti-IL-2 antibody (R&D Systems) followed by an anti-goat PE-F(ab′)$_2$ (Jackson Immuno Laboratories). Secondary and tertiary antibodies were used at 10 nM concentration in PBS with 3% nonfat milk, 0.05% Tween 20, and 250 nM mouse IgG Fc as a blocking agent. Incubations were performed as described for the IL-8 detection antibody with an extra wash step between the two antibody incubations.

Results

Generation of random and site-directed biotinylated IgG and Fab′ reagents

The four biotinylation procedures (Fig. 1) were carried out with three different antibodies, as described under Materials and methods: MAB9647 and MAB208, which bind to human interleukin-8, and MAB602, which binds to human interleukin-2. The oriented forms are denoted by the prefix "Or," and the random forms by "Ran."

Monitoring surface density and binding activity using SPR

To measure the surface density and surface activity of the various capture agents, we carried out an extensive series of surface plasmon resonance assays using BIAcore. From the SPR measurements we were able to calculate the maximal surface density and binding activity exhibited by the various capture agents analyzed in this study.

Fig. 3A shows an SPR sensogram for the deposition of SA onto the b-SAM surface. Biotinylated antibodies and their fragments can then be applied to this surface, as shown for Or-Fab′602. Subsequently, binding by the specific analyte, IL-2 in this case, can also be monitored. The binding of analyte is reversible and can be removed by brief acid pulses (pH 2.0 for 30 s in this case). After washing, analyte binding could again be monitored, and we observed no detectable activity loss after the washes. The deposition of capture agent was entirely dependent on the SA–biotin interaction as shown in Fig. 3B: pretreatment of the SA surface with biotin completely eliminated binding of Or-Fab′602.

Streptavidin could be consistently deposited at 3.7–4.0 pmol/cm^2 (2.2–2.4 × 10^{12} molecules/cm^2) according to the change in resonance units, which were converted to picomol as described [40] using a molecular

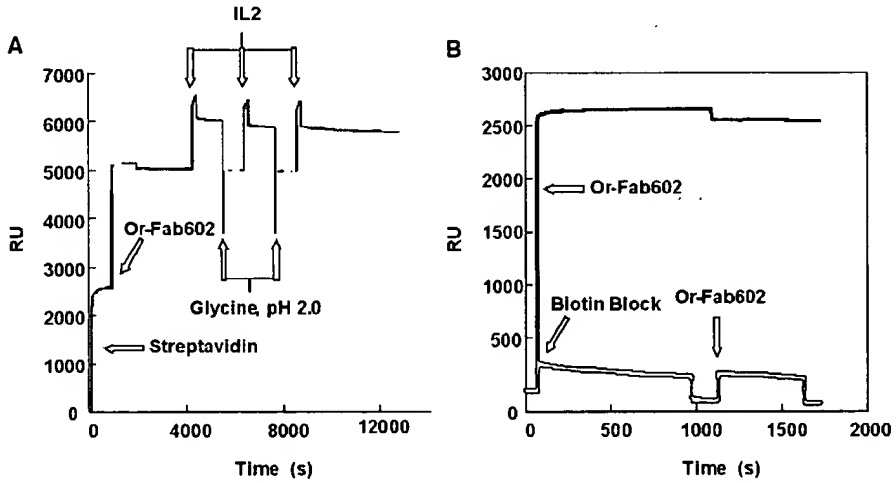

Fig. 3. Biotin-dependent deposition of streptavidin and biotinylated capture agents onto the b-SAM surface and the subsequent binding of analyte by the capture agent. (A) Typical sensogram from a BIAcore experiment which demonstrates the sequential loading of SA, Or-Fab′602, and IL-2 onto the b-SAM monolayer, as indicated by the arrows. Reactions were performed in PBS with 0.05% Tween 20 at room temperature. For the binding of SA (0.1 mg/ml stock) and Or-Fab′602 (50 nM stock) flow rates of 20 µl/min were employed. For IL-2 binding steps, reactions were carried out at 40 µl/min. To titrate the active sites, IL-2 was assayed at 600 nM so as to be at least >100-fold above the K_d (approximately 100 pM, data not shown). The capture surface was regenerated with a 30-s injection of glycine, pH 2.0, solution, as indicated by the arrows, to release any remaining analyte. The surface could be regenerated to nearly 100% activity as assessed by the SPR measurements. (B) After deposition of the typical amount of SA (not shown), 50 nM Or-Fab′602 was injected with or without a preinjection of 1 mM free biotin. Preinjection of free biotin blocked the loading of the capture agent.

weight of 60 kDa for SA. This density is only slightly lower than that measured for a two-dimensional SA crystal by electron crystallography (4.65 pmol/cm^2; [44]). Similar analysis was used to determine the density of bound capture agent. By varying the concentrations of analyte and allowing reactions to reach completion, we were able to titrate the active binding sites on the surface and assess the maximal amount of analyte that could be bound by each type of capture surface. This type of analysis was carried out extensively for the various capture agent forms generated for this study. By normalizing the deposition of capture agents and analytes to that of SA, we were able to compare the effects of the various linkage types and orientations on the surface density and binding properties of the three different antibodies and their Fab′ counterparts.

Effects of the various coupling strategies on surface density and binding activity for the different antibodies and their Fab′ counterparts

The graphs in Fig. 4 summarize the various surface densities and binding activities seen for the linkage methods used for this study. Specifically, we find the Or-Fab′ in all three cases to exhibit the highest surface densities, averaging almost 1 Fab′ molecule per SA molecule on the surface (Fig. 4B). Because the SA density approaches that of a two-dimensional crystal monolayer, the same can be said of the Fab′, since its molecular weight (50 kDa) is only slightly lower than that of SA (60 kDa). Also, the analyte-binding activities of the Or-Fab's are higher than the other species, with at least 70% of the binding sites being active (Fig. 4C). As a result of this high capture agent density and activity, the capacity of this surface for the analytes is also remarkably high (72–99% of that for streptavidin, Fig. 4A). This corresponds to a captured analyte-binding density of up to 4 pmol/cm^2 (2.4×10^{12} molecules/cm^2). It should be noted that in the experiments shown here, the analytes have a lower molecular weight than do the capture agents. For larger analyte molecules, lateral packing effects would be expected to reduce the number of capture agents that could be simultaneously occupied.

The effects of orientation are evident by comparing the Or-Fab's to the Ran-Fab's. The overall beneficial effect of Fab′ orientation on surface activity (Fig. 4A) is 5.6-, 4.8-, and 1.8-fold for Fab's 602, 208, and 9647, respectively. The high degree of variation in these numbers results not from inherent measurement errors (which are much lower, as represented by the error bars), but from differences between particular antibodies. By contrast, different Fab′ sequences respond very

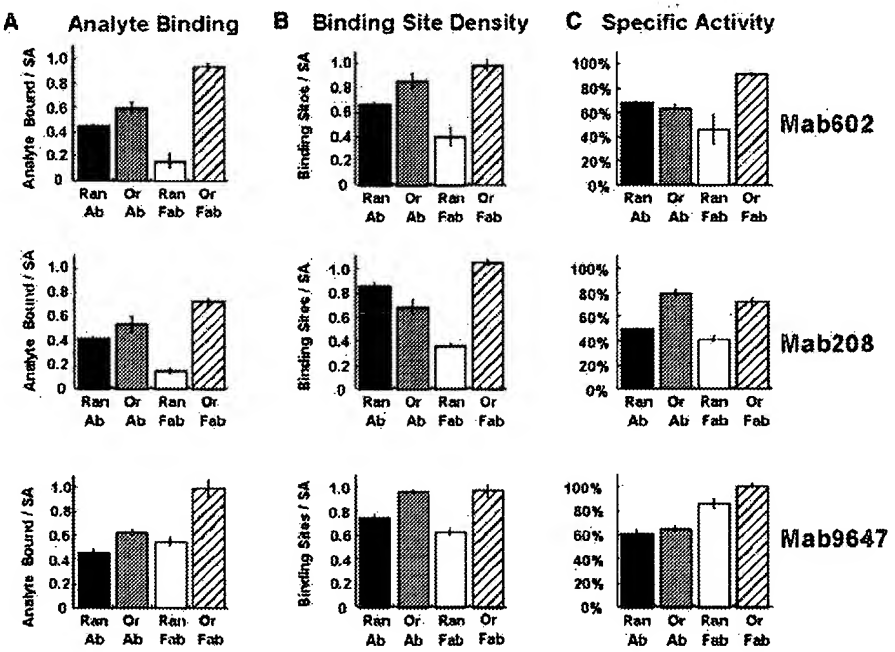

Fig. 4. Effects of the various linkage strategies on binding site densities and binding activities. From BIAcore assays similar to those shown in Fig. 3, the surface densities of the various capture agents and their respective percentage activities were determined, as described under Materials and methods. To standardize the data from each experiment, ratios of capture analyte/SA (A) and analyte/(No. of binding sites) (specific activity; (C)) were computed from the surface coverage values to compare the relative binding site densities and activities for the various types of capture agent forms. A valency of two for the full-length antibodies and a valency of one for the Fab′ fragments were assumed in calculating the binding site/SA ratio in (B). Specific activity is defined as the density of bound analyte (A) divided by the binding site density (B). To saturate the active sites, 100 nM analyte concentrations were employed for Mab208 and Mab602 while 1 μM analyte concentration was used for Mab9647.

reproducibly in comparison with each other when specifically oriented onto the SA surfaces.

Specifically orienting the full-length antibodies was found to provide a modest increase in total analyte-binding activity (33% on average) as compared to that of randomly oriented antibodies. The effect of orientation may not be as important for these large, flexible molecules. Overall, the oriented Fab′-derivatized surfaces bound 49% more antigen than the oriented IgG-derivatized surfaces.

Effects of orientation using a PLL–PEG–biotin-based surface incorporated into a protein microarray system

The importance of random versus oriented attachment of capture agents would be expected to depend on the nature of the underlying surface. We investigated the different forms of capture agents based on MAB208 immobilized onto a PLL–PEG–biotin monolayer surface that has been described previously [42,43]. In this surface, the biotin groups reside at the distal ends of long PEG chains that are grafted onto a polylysine polymer that is electrostatically adsorbed to an underlying titanium dioxide surface. This surface is more compatible with fluorescence-based immuno-sandwich assays since it lacks the gold layer (which can quench fluorescence). Furthermore, this surface has been utilized for the construction of protein microarrays consisting of 50-μm-diameter protein spots at a density of 10,000 features per cm^2. Proteins are patterned at this density by using a high-precision dispensing robot [42]. Fig. 5A shows a pseudocolor image of an IL-8-binding assay with the four forms of capture agent based on MAB208. The arrays were incubated with 100 nM fluorescently labeled IL-8, washed, and then read on a confocal fluorescent scanner. Each form of capture agent is replicated 25 times in a 5 × 5 array. The array scans are shown in Fig. 5A and quantification of the fluorescent intensity is shown in Fig. 6A. The specifically oriented capture agents give dramatically higher signal than the randomly oriented species. Quantification of the results shows that the oriented antibody provides about 10-fold higher signal than the random antibody. The oriented Fab′ provides about 5-fold higher signal than the randomly bound counterpart and about 1/3 of the signal as compared to the oriented antibody.

Immunoassays for measuring protein abundance are typically executed using a sandwich approach, in which one antibody (the capture agent) is immobilized, and detection occurs by the binding of another, labeled antibody to the captured analyte. This method avoids the need to chemically label analytes for detection. We com-

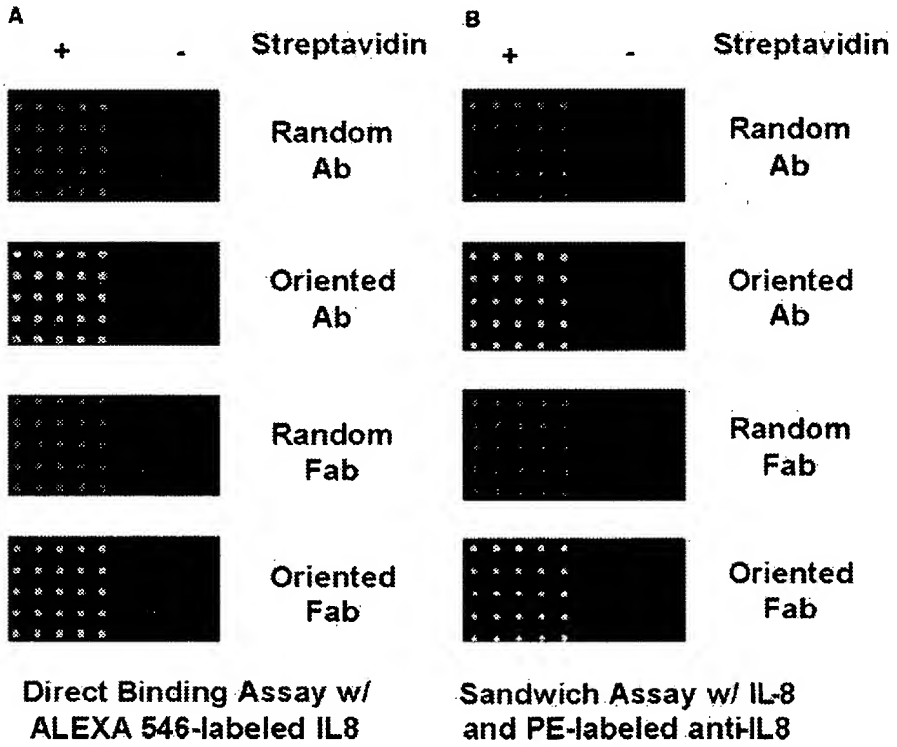

Fig. 5. Effects of the various linkage strategies on MAB208 capture agent performance on the PLL–PEG–Biotin microarray matrix. (A) Direct binding assay using 100 nM ALEXA 546-labeled IL-8. Adjacent to the 5 × 5 capture set is a control set of pillars on which were deposited PLL–PEG without SA. The image was taken in a GSI Lumonics ScanArray 5000XL with a laser setting of 80 and a photomultiplier tube (PMT) setting of 80. (B) Sandwich assay using 20 nM unlabeled IL-8 and 10 nM PE-conjugated anti-IL-8 detection antibody. This image was taken using a laser setting of 70 and a PMT setting of 70. For both the A and B, all assays were conducted on a single chip and scanned simultaneously.

pared the results of this type of assay to the detection of directly labeled IL-8 (Figs. 5B and 6B). The results are very similar to the direct binding measurement, with the specifically oriented capture agents performing 5- to 10-fold better than the randomly oriented agents. As in the direct assay on the same surface, the oriented antibody produces higher signal than the oriented Fab′ fragment, but the difference is not as dramatic in this case.

All of the experiments described so far were performed with analyte concentrations exceeding the dissociation constants for the capture agent–antigen interaction, which is approximately 100 pM for both Mab208 and Mab602 (as determined on low-capture-density BIAcore CM5 chips; data not shown), and were therefore designed to measure the capacity of the various surfaces. Under these conditions, slight differences in the affinity of the various capture agents due to steric effects, for instance, could be masked. For this reason, we repeated the IL-8 sandwich assay with low (100 pM) analyte concentrations (Fig. 7). Under these conditions, the specifically oriented agents still significantly outperform their randomly oriented counterparts. However, the relative amount of captured antigen in the two types of specifically oriented binding agents is reversed in comparison to the assay at higher concentrations. At 100 pM analyte, the specifically oriented Fab′ provides threefold more signal than the specifically oriented antibody. The most likely explanation for this reversal is

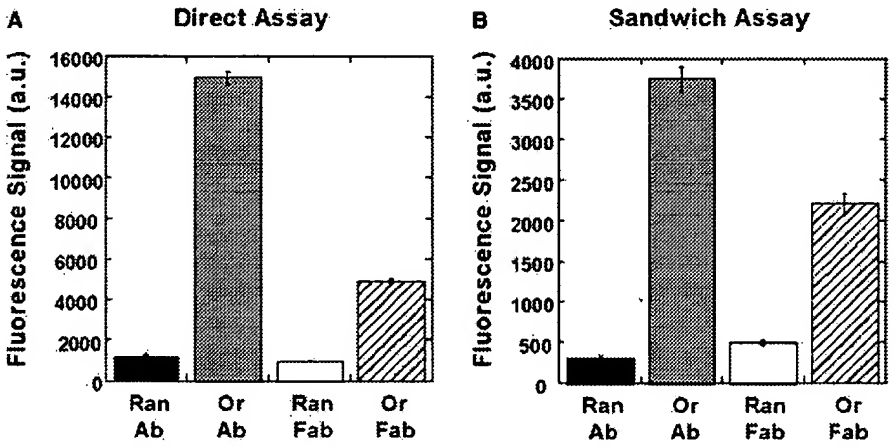

Fig. 6. Quantitation of the MAB208 direct binding and sandwich assays. (A) Plot of the average fluorescence intensities for the various MAB208 capture agent forms from the direct binding assay in Fig. 5A. Data represent the average intensity ± the standard error of the mean (SEM; $N = 25$). (B) Plot of the average fluorescence intensities for the various MAB208 capture agent forms from the sandwich assay in Fig. 5B. Data represents the average total intensity ±SEM ($N = 25$). For both A and B, all assays were conducted on a single chip and scanned simultaneously.

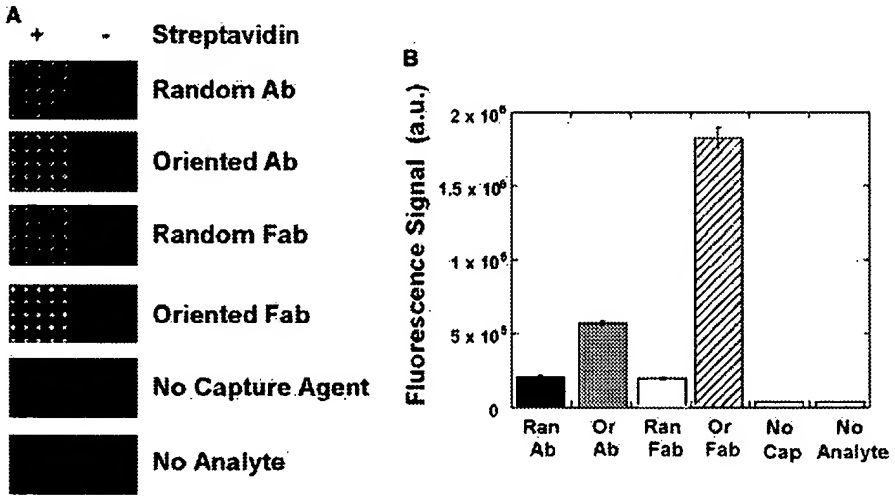

Fig. 7. Comparison of the four MAB208 capture agent forms on the PLL–PEG–biotin microarray surface at 100 pM IL-8. (A) Binding of 100 pM IL-8 to the various capture agent forms of MAB208 as monitored via a sandwich assay using 10 nM PE-conjugated anti-IL-8. (B) Average intensities ±SEM ($N = 25$) for the data in A.

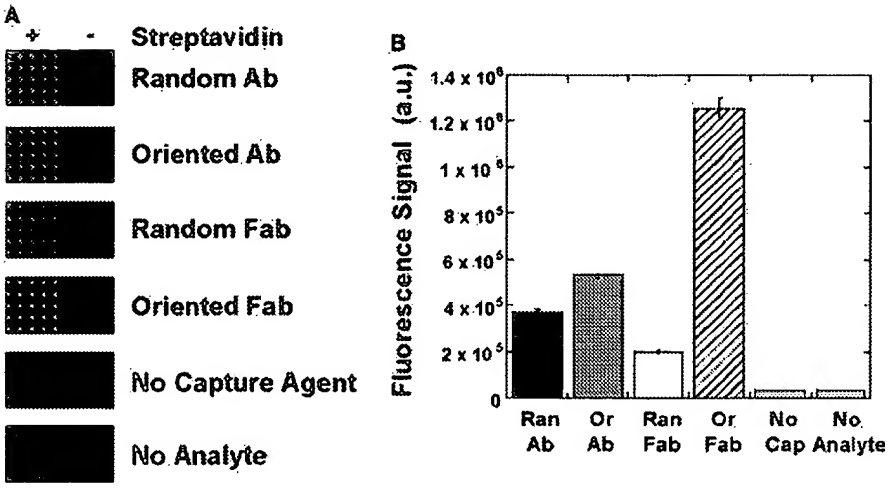

Fig. 8. Comparison of the four MAB602 capture agent forms on the PLL–PEG–biotin microarray surface at 1 nM IL-2. (A) Binding of 1 nM IL-2 to the various capture agent forms of MAB602 as monitored via a tripartite sandwich assay using 10 nM goat anti-IL-2 and 10 nM PE-conjugated donkey anti-goat IgG. (B) Average intensities ± SEM ($N = 25$) for the data in A.

that the oriented antibodies may have, in comparison to the oriented Fab's, a higher number of active antigen-binding sites per surface area but a lower average affinity for analyte, e.g., due to steric influences or damage of the antibodies from the periodate oxidation [45]. Under conditions of low analyte concentration (below the dissociation constants), the amount of captured analyte is highly dependent on the affinity of the capture agents, but under high analyte concentrations (above the dissociation constants), small differences in the affinities have a less significant effect on analyte binding than does the overall surface capacity. The actual dissociation constants of the immobilized capture agents are probably heterogeneous [20], and the affinity distributions probably differ between the antibody and the Fab′ surfaces. Such differences could account for the analyte concentration-dependent nature of the relative performance of these two surfaces.

We performed a similar comparison with the IL-2 capture agents (Fig. 8). The trend is very similar to that observed for IL-8 (Fig. 7), with the specifically oriented Fab′ fragment outperforming the other types of agents.

It should be noted that in these experiments, the amount of biotinylated capture agent is vastly greater than the available biotin-binding sites on the features, so the difference in antigen-binding capacity in different capture agent forms is not due to slight differences between samples in the concentration of biotinylated capture agent that was applied to the chip surface. Rather, these differences should reflect some combination of the number of immobilized capture agents per feature and the activity of these immobilized agents. We cannot say which of these two factors contributes more to the observed difference in the amount of captured analyte in the specifically oriented and randomly immobilized capture agents.

Discussion

We tested four different types of capture agents (randomly immobilized antibodies, specifically oriented antibodies, randomly immobilized Fab's, and specifically oriented Fab's) on two different types of streptavidin-coated surfaces. The b-SAM surface consists of biotin groups attached to an oligoethylene glycol-containing self-assembled alkanethiol monolayer on gold. This surface was used to measure the absolute packing density of capture agents and their specific activity under conditions of high analyte concentration. The PLL–PEG surface consists of biotin groups attached via long PEG units grafted onto a poly-L-lysine backbone that is electrostatically adsorbed to a titanium dioxide surface [42]. This surface was used to determine the relative, rather than the absolute, amount of analyte captured by the different capture agent forms, at low analyte concentrations.

The specifically oriented capture agents consistently outperformed the randomly affixed agents, but to different degrees depending on the antibody sequence, the type of surface, and whether an antibody or a Fab′ was used. Surfaces derivatized with Fab′ fragments showed 1.8- to 5.8-fold higher binding capacity when the Fab′ was specifically oriented as opposed to randomly attached to the b-SAM surface. The same comparison on the PLL–PEG surfaces also showed an increased capacity on going from random to specific immobilization (5- to 10-fold). The benefits of specifically oriented attachment were less dramatic for the full-size antibodies on the b-SAM surface (33% better than randomly oriented on average), but very significant on the PLL–PEG substrate (6- to 10-fold). The fact that oriented attachment is more important on the surface with long PEG tethers to the capture agent (PLL–PEG) than on the surface with short PEG tethers

(b-SAM) was unexpected, and we do not have an explanation for this finding.

The Fab′ fragments consistently packed more densely on the b-SAM surface when specifically oriented than when randomly attached (twofold on average). The average packing density was 4.0 pmol/cm^2 (2.4×10^{12} molecules/cm^2), which corresponds to 1 Fab′ per SA. Since the SA surface density is 85% of that found in two-dimensional SA crystals [44], and the Fab′ is only slightly smaller than SA (50 kDa vs 60 kDa), these surfaces are very tightly packed with Fab′. The Fab's retain almost complete activity (average 92%) when oriented in this way. To our knowledge, this is the highest reported density of active, protein-based binding agents on a planar surface.

There have been numerous reports that specific orientation of antibodies and Fab′ fragments can increase the binding activity of these proteins compared to their randomly attached counterparts [13,19–21,25,31,46]. These benefits are usually counterbalanced by the fact that the procedures for specifically orienting proteins usually result in a lower surface coverage than do methods for direct, random surface attachment [13,19–21,46]. Using the densely coated SA-derivatized surfaces described here, it was possible to achieve the twin goals of maximizing both the packing density and the activity of the capture agents. Such surfaces are ideal for incorporation into antibody microarrays for measuring the concentrations of low-abundance proteins in complex biological mixtures.

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